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# The Role of Serum Histones in Canine Heat Stroke

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# The Role of Serum Histones in Canine Heat Stroke

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By

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**Table of Contents**

**Abstract:**

Rising temperatures all over the world has correlated with more frequent heat stroke related injuries and death. This statistic not only applies to humans, but to canines as well, who have similar body temperature thresholds. Recent studies have demonstrated that serum histones, released after cell death from heat stroke, play a role in heat stroke related injuries and death. This proposal aims to determine the severity of the effects caused by serum histone release after heat stroke by exposing selected canine cell lines to cell lysate and purified histones H2A, H2B, H3, and H4, which have been found to be associated with heat stroke injuries. Effectiveness of the histones will be determined by measuring the levels of apoptosis, NETosis, and necrosis in the cells, as well as the expression levels of heat shock proteins. Further research will also be done to determine whether toll-like receptors present on the cell surface are responsible for the mechanism utilized by serum histones to damage tissue in the body.

**Introduction:**

In the United States alone, heat stroke has been the cause of death for more than 9,000 people since 1979, with a major peak in 2006, one of the hottest recorded years in history. Heat stroke can be defined as an elevated core body temperature above 40°C, leading to the disfunction of the central nervous system. In more specific terms, thermoregulation failure occurs along with an exaggerated acute-phase response, possibly coupled with altered expression of heat-shock proteins. In some victims multiorgan injury results from the interactions between the cytotoxic effect of the heat and a coagulation response from the hosts' body (Bouchama et al. 2002). Canines have also been found to be susceptible to heat stroke; it was found that dogs whose rectal temperatures exceeded 43°C exhibited clinical, hematological, biochemical, and anatomopathological manifestations nearly identical to those of humans who experienced heat stroke (Shapiro et al. 1973). The threshold temperature of humans and canines is very similar allowing for a bridge of the typical gap between human and veterinary medicine. Currently, there is no single cause that has been pinpointed for multiorgan dysfunction and eventual death after heat stroke. However, research has shown that serum histones may exacerbate the severity of these symptoms after an episode (Bruchim et al. 2017). This proposal will aim to determine how histones, specifically serum histones, play a role in canine heat stroke and the detrimental after effects it has on the body. Examination of these biomarkers will help lead the way into further research on how to prevent these catastrophic results by manipulating the histones in a way that allows for a targeted therapy.

## Heat Stroke

Heat stroke is defined by patients' symptoms that present themselves at the time of clinical admission, which includes, but is not limited to, central nervous system abnormalities (coma, seizures, delirium) and sever hyperthermia (an internal core temperature of above 40°C). Many times heat stroke can cause death, and about 30% of heat stroke survivors experience permanent damage in neurological and peripheral tissue function. For years it was thought that the severity of hyperthermia in heat stroke was the primary cause of mortality, however recent experimental evidence suggests that the mechanism of death is much more complex. Specifically heat cytotoxicity, coagulation, and systemic inflammatory response syndrome (SIRS) are all interconnected and lead to damage of the gut and other organs after heat stroke (Figure 1) (Leon et al. 2010).

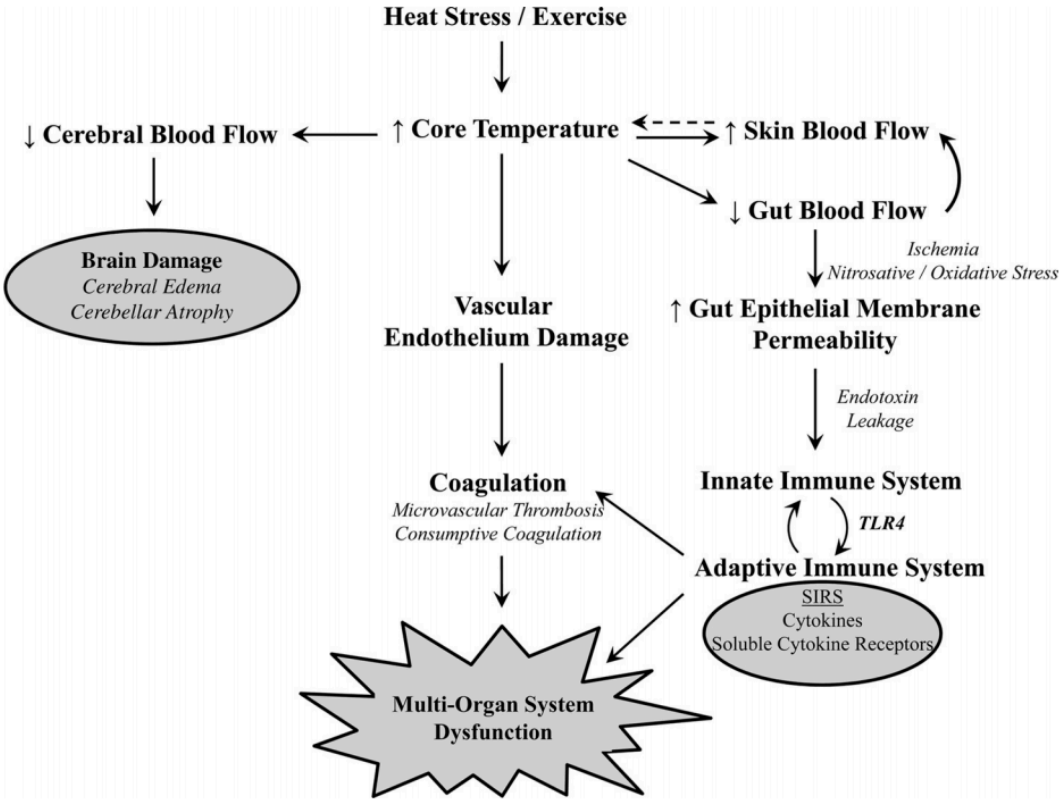


**Figure 1.** Summary of heat stroke pathophysiological changes that eventually lead to multi-organ failure (Leon et al. 2010).

Epidemiological studies have shown that multi-organ damage caused by heat stroke continues to manifest in patients even after treatment, increasing the risk of mortality in the coming months after the onset of the illness. The systemic inflammatory response syndrome is a response to a bacterial infection that follows damage to the gut and other organs after prolonged reductions in splanchnic blood flow. This causes the gut to become "leaky" and allows for bacteria that typically live in the lumen to easily cross through the membrane and enter the systemic circulation. In addition, the liver has been seen to play an important role in endotoxin clearance, linking liver damage and circulating endotoxins after heat stroke in patients (Leon et al. 2010). Not only has heat stroke been found to affect the gut and liver, it has also been linked to kidney, hematological, vascular endothelium, and brain damage (Lumlertgul et al. 1992).

## Heat Shock Proteins

Looking to the cellular level of heat stroke, nearly all cells when exposed to higher temperatures respond by producing heat-shock proteins. This mechanism occurs during heat stress, when heat-shock transcription factors bind to the heat-shock element, leading to an increased rate in transcription of heat-shock proteins. The increase in the level of heat-shock proteins allows the cell to induce a state of tolerance to the next stage of the illness, heat stroke, and allows it to survive during this state of trauma (Bouchama et al. 2002). Even though heat shock proteins are expressed when cells are exposed to elevated temperatures, there can be alterations in the expression of these proteins causing cells to eventually die, as they cannot withstand these temperatures in the absence of the proteins. Heat directly causes tissue injury in cells, however, the temperature at which this happens in mammals varies by species. Cells also have been found to secrete cytokines when under this environmental stress. Specifically, plasma levels of inflammatory cytokines and anti-inflammatory cytokines are elevated in patients with heat stroke. An imbalance of inflammatory and anti-inflammatory cytokines can result in an inflammation-related injury (Bouchama et al. 2002). When a cell dies, the internal contents of the cell can leak into the bloodstream. Typically, phagocytes will protect the body from any harmful cell content that was released after the cell died. However, extracellular histones have been found in elevated levels after a patient undergoes heat stroke, and these histones are believed to play a role in systemic inflammatory and toxic responses in the body after the illness.

## Histones

Histones are typically found in the nucleus of eukaryotic cells, where they act as a unit of chromatin structure, allowing nucleosomes to be constructed as DNA wraps itself around the

histones for organization. They also play a role in gene regulation, and many DNA-associated processes, such as transcription, replication, and DNA damage repair (Chen et al. 2014). There are six main histone types, which include core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5). Each of the core histones are made up of two domains: the globular region, which is characterized by the presence of a histone fold domain, and the N-terminal tails, which aid in transcriptional activation, silencing, and chromatin assembly (Figure 2).

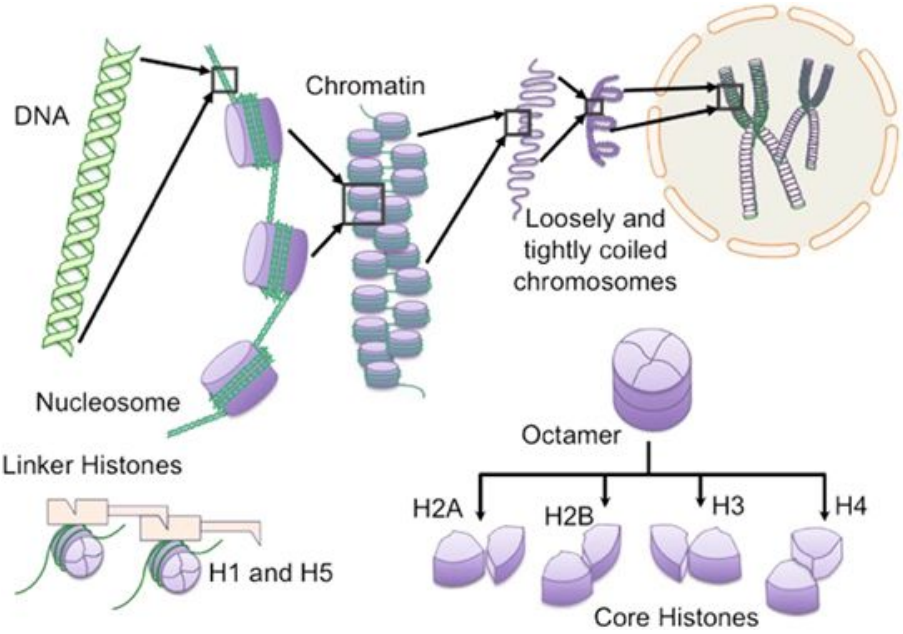


**Figure 2.** An overview of the structure and function of the different histones (Silk et al. 2017).

## Serum Histones

When histones enter the bloodstream after a cell has died, it is now classified as a serum or extracellular histone. Histones may be released into the extracellular space in 3 possible forms: freely, bound to DNA as a nucleosome, or as part of a neutrophil extracellular trap (Silk et al. 2017). Recently it was discovered that extracellular histones are toxic and produce a pro-inflammatory response in the body of the patient. In one study, free histones were intravenously

administered to mice. The results demonstrated that these histones are in fact toxic, as the mice died within minutes upon injection (Xu et al. 2009). Histones are typically released into the blood stream in patients or animals who have cancer, inflammation, and infections, suggesting that these histones play a role in diseases. It was determined that serum histone levels in canines that had experienced heat stroke were elevated, which was shown by determining the levels of serum histones in dogs that had not been affected by heat stroke, dogs that had heat stroke but survived, and dogs that did not survive a heat stroke attack. The results indicated that histone levels had a positive correlation with the severity of the disease, as there were significantly higher levels of serum histones in non-survivors and dogs with hemostatic derangement (Bruchim et al., 2017) (Figure 6). The six histone types are typically detected at the cell surface of immune cells, cerebellar neurons, Schwann cells, and microglia when the body has been under stress, such as heat stroke. Once the body undergoes this trauma, the histones are released into the bloodstream typically by either NETosis, necrosis, or apoptosis. NETosis is a unique form of cell death that releases neutrophil extracellular traps (NETs) containing histones from immune cells. This mechanism is mediated by protein arginine deaminase 4, which facilitates citrullination of H3 and subsequent chromatin degradation (Silk et al. 2017). On the other hand, cells undergoing necrosis typically release free histones into the extracellular space. This involves an uncontrolled rupture of the plasma membrane, releasing most of the components of the cell, including intra-nuclear proteins. Apoptosis, known as the “silent death”, does not disrupt the plasma membrane, thus histones leak from the membrane blebs of these cells.

## Effects of Serum Histones

Extracellular histones have been found to play a role in numerous diseases, both infectious and non-infectious. These diseases include, but are not limited to sepsis, trauma, thrombosis, stroke, acute kidney, lung disease, and many more. The mechanism by which these serum histones play a role in toxicity differs depending on the disease, as histones affect the body in many different ways (Allam et al. 2014) (Figure 3).

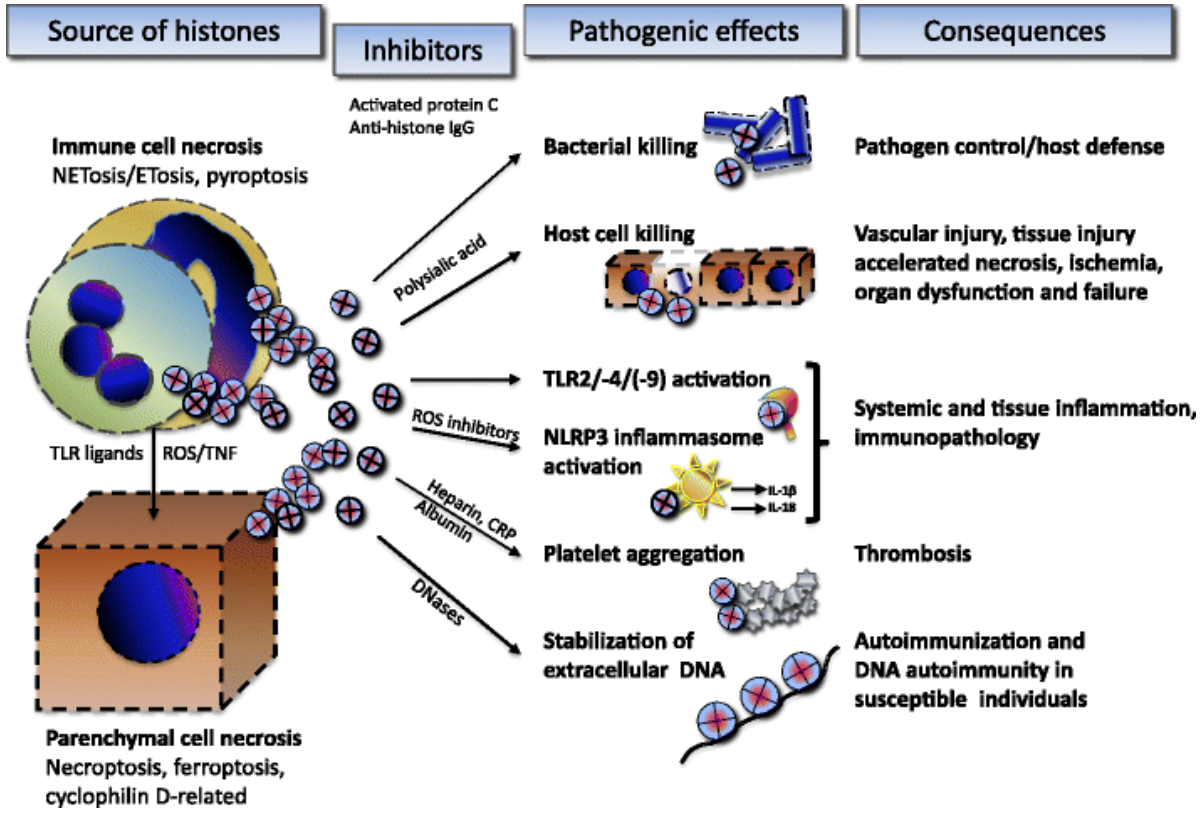


**Figure 3.** An overview of how serum histones trigger tissue injury and inflammation in the body. There are numerous ways in which extracellular histones produce negative effects on organs and tissues (Allam et al. 2014).

For example, in sepsis, a systematic inflammatory response syndrome caused primarily by bacterial infections, the extracellular histones act cytotoxically to facilitate microvascular dysfunction. This stands true even though histones have been found to have strong antimicrobial properties, which should alleviate sepsis, rather than enhance disruptive effects (Hirsch et al.

1958). Histones have also been found to play a key pathological role in trauma patients, where high levels of serum histones have been positively correlated with severe complications. *In vitro*, serum histones lead to the production and secretion of cytokines, stimulate NET formation, and increase calcium influx in both immune and endothelial cells, which helps facilitate serum histone cytotoxicity. Similarly, *in vivo* studies have found that serum histones accelerate cytokine release, endothelial damage, coagulation activation, and lung injury following a trauma (Chen et al. 2014). The mechanism of tissue injury and inflammation in the body caused by extracellular histones is well understood by researchers and is thought to be one of the main mechanisms by which extracellular histones act.

**Inflammatory Response**

Serum histone levels are significantly elevated in animals that have experienced liver, kidney, lung, and brain injuries, all of which are common in heat stroke. This suggests that histones may play an important role in inflammation and injury. It is thought that serum histones cause these detrimental effects in the body through the binding of toll-like receptors to induce the production of pro-inflammatory cytokines, which accelerate the inflammatory responses and tissue injury (Figure 4).

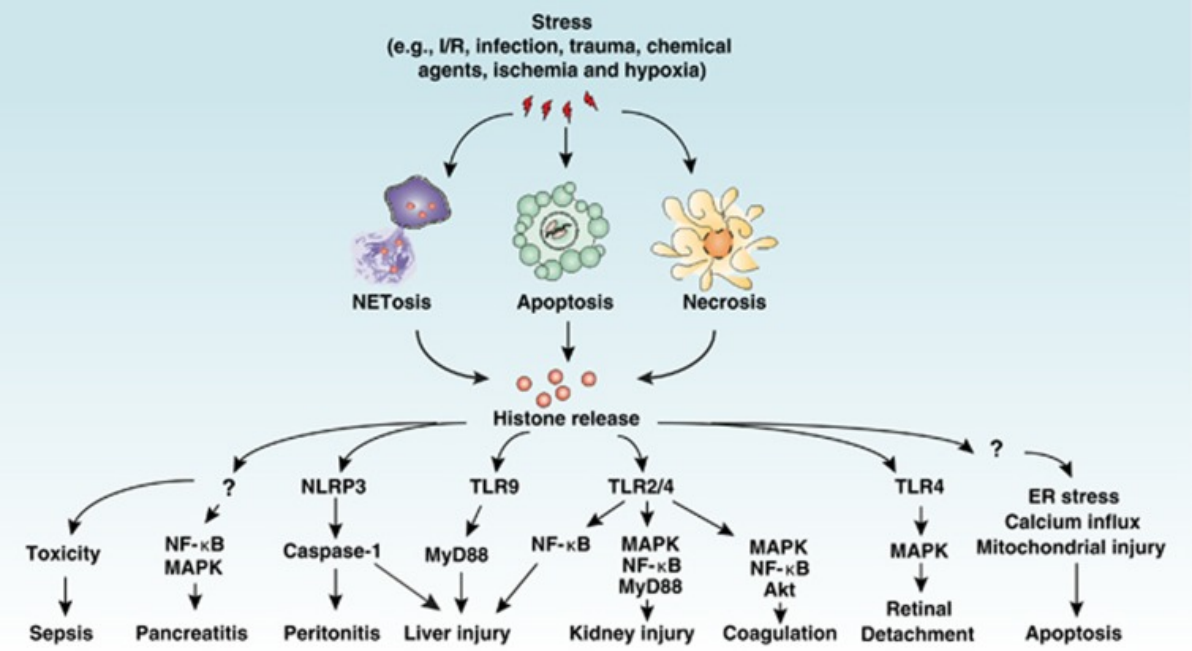


**Figure 4.** A complete overview of how serum histones play a role in many diseases in the body (Chen et al. 2014).

The specific toll-like receptors involved include TLR2, TLR4, and TLR9. Because these histones interact directly with the toll-like receptors on cells, they can be categorized as acting as a damage associated molecular pattern (DAMP). DAMPs are biomolecules that can initiate and perpetuate an inflammatory response in the body. In previous work, mice with knockout TLR9 protected against histone-mediated liver injury (Huang et al. 2017). These results were based off the evidence that DAMPs produced after ischemic injury function through the use of TLR9 to produce inflammation, thus the knockout mice had more mediated symptoms than the control mice. These results support the idea that serum histones work using toll-like receptors, however, it does not directly correlate with heat stroke. They also did not test for the effects of TLR2 or TLR4 on serum histone activity. However, serum histones are not thought to work alone in promoting tissue injury and inflammatory responses. It has also been hypothesized that the histones work with other pro-inflammatory agonists through “cross-talking” to damage cells

(Chen et al. 2014). Overall, tissue damage plays the greatest role in heat stroke related deaths, as this eventually leads to multi-organ death in patients. Extracellular histones have been found to damage numerous organs, most of which are involved in multi-organ failure after heat stroke (Figure 5).

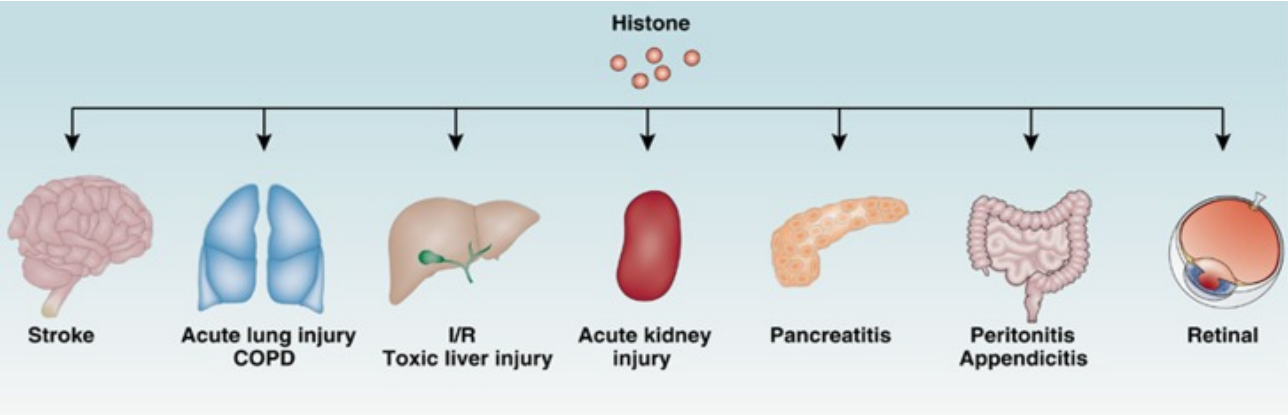


**Figure 5.** Tissue and inflammation targets of serum histones (Chen et al. 2014).

## Heat Stroke in Canines

Only recently have studies been emerging linking heat stroke and extracellular histones, however this research is on the rise as heat stroke is becoming a much more prevalent disease due to the warming temperatures of the Earth. Just as heat stroke in humans is on the rise, so too is the prevalence of heat stroke in canines. Similarly to humans, severe complications associated with heat stroke in canines include rhabdomyolysis, acute kidney injury, acute respiratory distress syndrome, and disseminated intravascular coagulation. Mortality rates in both humans and canines are around 50% after heat stroke, even with proper treatment and appropriate body cooling. With this recent surge of research investigating the role of histones in heat stroke, it has been proposed that histones can act as biomarkers for the severity of heat stroke in canines (Bruchim et al. 2017). Since heat stroke is a severe systemic inflammatory response syndrome

that mimics sepsis very closely, it was thought that serum histone concentrations would change in a similar way for both conditions. In a completed study, serum histone levels in 16 dogs with naturally occurring heat stroke were compared with 7 healthy control dogs. It was found that canines who died from heat stroke had significantly higher levels of serum histones than those that did not suffer from heat stroke (Figure 6).

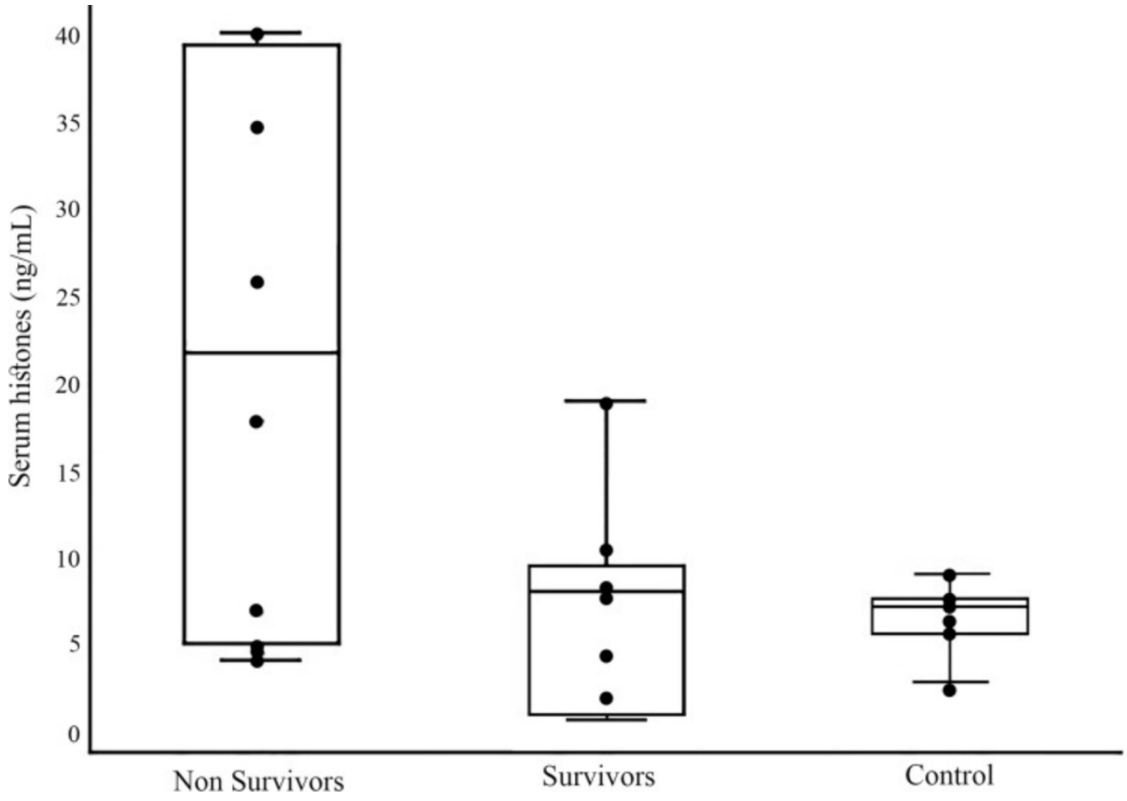


**Figure 6.** Median total serum histones concentration at presentation to the hospital in ten non-survivor, six survivors, and seven healthy controls dogs with naturally occurring heat stroke. Median total serum histones are significantly increased ($p = 0.043$) in all dogs with naturally occurring heat stroke compared to their level in seven healthy dogs. Median total serum histones in non-survivors was significantly ($p = 0.049$) higher among non-survivors compared to survivors (Bruchim et al. 2017).

This positive correlation between serum histones and heat stroke in canines, along with the increase in more severely affected patients, implies that these histones play a role during heat stroke. That specific role has not yet been discovered, however it is thought that it plays some

part in coagulation, oxidation, and inflammation in the body. It is also unclear how great of an effect these histones have on the body after heat stroke, as well as how they specifically work to produce these detrimental results. This proposal will focus on determining the quantity of serum histones in the body after heat stroke, the mechanism by which serum histones exert their effects, and what can be done with this new information to help protect against heat stroke mortality and injuries, specifically in canines. With this information, further research can be done in humans with heat stroke, as both canines and humans have been found to react similarly when diagnosed with heat stroke.

**Experimental Design**

Serum histones have been found to have a detrimental effect on the body after a heat stroke attack, however, the cause of these effects has not yet been confirmed. This experiment will be broken up in to two aims. The first aim will help to determine how much of an effect serum histones have on the body after a heat stroke attack, compared to the other components of the cell, and will narrow down which histone and which part of the histone is causing this damage. The second aim will examine the mechanistic aspects of serum histones, determining if a certain receptor on cells is critical for the histones to exert these effects.

**Aim 1**

**Lysing Cells with Heat**

The first experiment would involve exposing certain canine cell lines to high heat in order to model what typically happens in the body when heat stroke occurs. The specific cell

lines that were chosen for this experiment are a purified hematopoietic cell line, obtained from the Fred Hutchinson Cancer Research Center, a canine kidney cell line, MDCK NBL-2 from the American Type Culture Collection, and a lung tumor, COS_1003 from the American Type Culture Collection. The canine hematopoietic cell line was chosen as heat stroke has been found to affect coagulation in patients after an attack, and thus blood cells would be a strategic marker to measure the release of serum histones (Bruchim et al., 2017). The canine kidney cell line was also chosen due to the fact that the kidney has been found to be severely damaged after heat stroke, prompting the idea that serum histones may attack the cells of the kidney (Leon et al. 2010). Similarly, the lung tumor cell line was chosen as the lungs are a common target of extracellular histones, leading to severe injury, and thus are a practical target for this experiment (Cheng et al. 2014). These 3 cell lines will be exposed to 2 different temperatures: 40°C and 43°C, the typical threshold for heat stroke in humans and canines, respectively. There will also be a control temperature, around 38°C, the typical body temperature of canines. The cells will be exposed to these temperatures for 10 hours, being sampled every 30 minutes over a 10 hour period to assay for cell damage, specifically apoptosis, NETosis, or necrosis. Flow cytometry will be utilized as a method to analyze these cells. Cell death will also be quantified by determining the levels of caspase, Annexin V, Bct2, TNFR, PARP, and phosphatidyl serine assays (Alberts et al. 2002).

In the first part of the methods, where the cell lines were solely exposed to high temperatures, we would expect to see most of the cells lyse. In the control temperature, no cells would be expected to lyse due to the surroundings. We would also expect to see a combination of cell death mechanisms. However, apoptosis would likely be the most common method utilized by the cells, as apoptosis is more prevalent than NETosis or necrosis in this temperature range

(40-43°C) (Bouchama et al. 2002). However, some cells will undergo these other modes of cell death, as they have been identified in previous research regarding histones and heat stroke.

**Exposing Cells to Lysate in the Absence of Heat**

Looking at previous research, it can be predicted that many of the cells will be severely damaged, if not completely lyse when exposed to the increased temperatures (Chen et al. 2014). When the cells break open, the full content of the cell, including proteins, organelles, intracellular fluid, and histones will be released. We will prepare extract from the heat shocked cultures. The entire cell culture will be collected, centrifuged, and the pellet containing the intact cells will be discarded. The supernatant (cell lysate) will be collected and mixed with untreated cells of the same cell line. The cells will be incubated for 10 hours at normal temperature (38°C). Samples will be collected every 30 minutes and analyzed through the use of flow cytometry and cell imaging (BD Biosciences instruments). Time points will also be taken every 24 hours after the initial exposure for 3 days, as some symptoms of heat stroke only appear after the onset of the disease. The number of cells that have lysed or died by means of the mechanisms mentioned previously will be quantified. Also, the expression levels of heat shock proteins will be measured, as this will give insight into whether the lysate causes the expression of these proteins to increase. The levels of protein will be measured using Western Blot analysis. The expression of the protein will most likely increase with heat, but it is not known if histones, or other cellular contents can activate its expression. This will allow for a baseline to be established when comparing these results to those of the following experiment.

In this step of the experiment, because the cells were expected to lyse, their components would be released into the cell media, and thus it would be important to determine what part(s)

of this extract had the greatest damaging effect on the other cells. It was predicted that the cells exposed to this extract would be negatively affected, as they are typically not around the components that make up the interior of the cell. The concentration of cells that are killed will be compared to the controls and other experiments performed to determine whether the lysate severely impacts the cells it comes in to contact with. One way in which cell lysis often disrupts the cellular environment is by releasing endogenous proteases and phosphatases into the bloodstream. As a result, proteins are degraded, ultimately causing other cells to become severely damaged, or even lead to cell death (Walker 2009). However, to look specifically at how great a role histones played in this destruction of other cells, we needed to observe the effect of histones alone.

**Exposing Cells to Purified Histones in the Absence of Heat**

After generating a standard in the previous experiment for the cell lysate, we would then study the effects of histone proteins alone. Hematopoietic, kidney, and lung cells used in the previous 2 experiments will be exposed to growth media containing purified histones, and their effect on these cells will be assayed. The specific purified histones used will be canine H2A, H2B, H3 and H4. These histones were chosen as they have all been found in the bloodstream, with H3 and H4 being most abundant in cases leading to organ and tissue injuries (Hoeksema et al. 2016). Serum histones have the same structure as intracellular histones, so no modifications need to be made to the purified histones being used, however we will not be able to account for any post-translational modifications that may be added to the histones inside the cell prior to lysis. As it is unlikely that a sufficient number of purified histones will be obtained from canine cell lines, these histones will be expressed in bacteria followed by purification using an antibody.

However, it is unknown how much histone should be added in order to elicit cell death, thus varying histone concentrations will be used. There will also be parallel samples with combinations of the 4 different histones, including one sample with all 4 histones. It is predicted that nucleosomes will form in these samples. To remain consistent with the other experiments, these cells would be incubated for 3 days, with samples taken every 30 minutes for the first 10 hours and once every 24 hours after treatment. Cell samples will be analyzed via flow cytometry and compared to an untreated control in order to determine the degree of induction of cell death and heat shock pathways by the addition of purified histones. This experiment, along with the previous experiment will enable us to determine how much of a role histones play in damaging cells after heat stroke. Specifically, if this experiment produces effects equally severe to the previous experiment the damage can be attributed exclusively to histones. Otherwise, another component(s) of the cell contents is responsible for the death of other cells.

After exposing the 3 cell lines to purified histones, we expect to see around the same percentage or slightly less cells damaged or lysed than were found in the previous segment of the experiment. This would confirm the idea that histones play a major role in the severity of heat stroke, as it proves that they were the key reason for cell death. However, if they do not show similar numbers, this implies that another factor(s) in the extract is responsible for the damage being caused. If the experiment yielded results that were much lower than the previous experiment, this would disprove the hypothesis that serum histones are a primary causative factor in heat stroke. Even if the cell death was very low (<10%) in this portion of the experiment, this would still confirm that histones play some sort of role in heat stroke, but smaller than originally anticipated.

**Determining the Role of the Histone "Globular" Domain and Tail**

If we observe a response to histones in isolation, we will perform further experiments to determine the specific domain of the histone that is detrimental to the cells, as this would prove important for further research regarding serum histones and heat stroke. As stated earlier, histones are comprised of two main regions: the nucleosome core particle, also known as the "globular" domain, and the N-terminal tails. It is not yet known if either region/domain of the histone is more destructive than the other, or if both the core and tail are required to harm cells. Histone core domains are typically related to chromatin structure, while the tails are associated with nucleosome stability, and contribute to define the condensed state of the chromatin fibers (Mariño-Ramírez et al. 2005). In order to determine if there is a difference between the core and tail of the histone, the 3 cell lines used in the previous experiments will be exposed to recombinant proteins containing H3 and H4 lacking N-terminal tails, and recombinant histone tails, which will all be analyzed in a similar manner. In order to generate the recombinant proteins with H3 and H4 lacking tails, these proteins will be expressed and purified from bacteria. The tailless histones will be expressed and purified from bacteria using a $His_6$ tag, which will be removed via thrombin treatment (Iwasaki 2013). Once again, flow cytometry, cell imaging, and measurement of heat shock protein expression will be employed to determine if one region of the histone is causing more damage to the cells than another.

Regarding this experiment that looked specifically at histone cores versus histone tails damaging cells, it is thought that both portions of the histone could have an approximately equal role in this mechanism. This is because typically serum histones are comprised of both the "globular" domain and tail, so it would be thought that the histone would most likely need both parts to properly function, and further damage the cells and tissues that surround it. However, it

could also depend on the mechanism by which the histones damage the cell. If only a certain portion of the histone interacts with a receptor on the cell, only that domain will be needed. Another possible outcome could be the tail of the histone causing more cells to lyse and become damaged than the "globular" domain. This could occur as histone tails have been found to play an essential role in determining the binding partners for the histones, nucleosome stability, and for producing higher order structures (Hansen et al. 1998). However, without establishing the immune recognition of the histones and determining which part triggers the immune response, it is difficult to predict whether one domain plays a greater role than the other.

**Aim 2: Receptor-Based Mechanism**

The mechanism by which serum histones promote a pro-inflammatory response in the body is thought to be through activation of toll-like receptors present on the surface of macrophage cells. This in turn produces cytokines, which accelerates the inflammatory response and hence, tissue injury (Cheng et al. 2014). To begin to determine how to potentially block serum histones from damaging the cells around them, the mechanism by which they do so must first be elucidated. In order to do this, toll-like receptors TLR2, TLR4, and TLR9 would be deleted from the selected cells, where the receptors are typically found. This deletion would be done through the use of the gene knockout tool, Crispr Cas9. The mutated cells lacking receptors would then be exposed to the histones that showed a response in the previous experiment, most likely H3 and H4, and the effects on cell death pathways and heat shock protein expression would be examined as before. However, if the purified histones did not show a response in Aim 1, the mutated cells will be exposed to the cell lysate instead.

Based on previous research, select toll-like receptors have been found to play an important role in the detrimental mechanism of serum histones on the body after heat stroke, specifically TLR9. Thus, it is thought that by completely deleting either the TLR2, TLR4, and TLR9 receptors from the cells, histones would not be able to produce damaging effects on these cells, as they have no way of promoting a response in the cells. However, if the serum histones continue to produce cytoxic effects on the exposed cell lines without access to the receptors, the proteins must be utilizing another method of destruction, prompting more research to be done.

**Further Thoughts**

This new information will allow further research to be completed on serum histones as a potential causative element in the after effects of heat stroke. If we know what exactly causes the histones to be released after a heat stroke and the mechanism by which they damage tissues, future work can be done in order to neutralize these histones to prevent these detrimental outcomes. Histones can be neutralized by therapeutic drugs, which can either degrade the histones directly or inactivate the histones by use of antibodies (Allam et al., 2014). Anti-histone antibodies have been used previously to target extracellular histones, but if they obtain access to the interior of the cell it could potentially disrupt the DNA structure or function, causing harmful side-effects (Silk 2017). More studies will need to be performed to gain a better understanding of how these histones affect cells, and in turn, what type of therapy would best minimize the effects of histones following heat stroke in canines.

Future experiments may aim to synthesize a therapy that solely targets the serum histones. This could potentially be done by targeting caspases that are involved with apoptosis by creating transition state analogs that inhibit the mechanism of release for these histones from

cells. However, in order to manufacture these analogs, the method by which histones are released must be more thoroughly understood. Once such information is obtained and therapeutic targets identified, the effectiveness and specificity of these future therapies will need to be evaluated in both humans and canines.

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